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(54) Title: MONOMERIC ANALOGUES OF HUMAN INSULIN

(57) Abstract: Monomeric analogues of human insulin have a single substitution of the amino acid in 12th, 16th or the 26th position of the B chain of human insulin and may also have a terminal deletion in the B chain.

MONOMERIC ANALOGUES OF HUMAN INSULIN

Field of the Invention

This invention relates to novel monomeric analogues of human insulin (HI) obtainable by recombinant DNA technology.

5 Background of the Invention

Insulin is highly effective in treating insulin-dependent diabetes, and has been used clinically for nearly 80 years. With advances in DNA technology and the development of biotechnology industries, insulin extracted from animal pancreas is gradually being replaced by recombinant forms of human insulin, produced in
10 microbial systems. This trend is encouraged by two observations; the number suffering from diabetes mellitus is on the increase globally and the clinical dose required to treat them is in milligram (mg) quantities.

Currently, the organisms employed for the commercial production of recombinant human insulin are *E. coli* and *S. cerevisiae*. The expression levels in *E.*
15 *coli* are high but difficulties associated with downstream purification often lead to loss of yield. These difficulties are not encountered with *S. cerevisiae*, because the insulin produced is secreted into the culture medium, facilitating purification. However, the level of expression observed in this organism is low and difficult to increase.

Until recently, introduction of Lispro®, clinical preparations of human insulin
20 contained polymeric forms of insulin which are slow-acting. Monomeric forms of insulin, as described in US-A-5618913, by contrast, are relatively fast-acting and mimic more closely the natural situation. They therefore demonstrate a great potential for clinical application. A commercial monomeric insulin, available as Lispro®, comprises inversion of amino acids 28 and 29 of the B chain of human insulin, and
25 may be abbreviated as B28Lys,B29Pro.

Kristensen *et al*, J. Biol. Chem. 272(20):12978-83 (1997), discloses alanine substitution at various positions on the insulin molecule, including B12, B16 and B26. A single substitution with Ala affected the binding activity of the resultant insulin analogue in certain cases.

30 Wang *et al*, Biochem. Mol. Biol. Int. 39(6):1245-54 (1996), discloses B12Thr, i.e. an insulin analogue in which the 12th amino acid of the B-chain of human insulin (Val) is substituted by Thr. Again, an effect on binding activity was observed.

EP-A-0046979 discloses des-B30 derivatives of human insulin.

EP-A-0291863 discloses des-B1 derivatives of human insulin.

Summary of the Invention

According to the present invention, novel human insulin analogues are monomeric variants of B12Thr, B16Ala and B26Ala; the latter have not previously been recognised as monomeric. In addition to replacement of any or all of the 12th, 16th and 26th amino acids on the B-chain, such that the analogue is monomeric, the B-1 and/or B-30 terminal amino acids may be absent. The term "insulin analogue" as used herein means a compound having a molecular structure similar to that of human insulin, including disulphide bridges between A7Cys and B7Cys and between A20Cys and B19Cys, and an internal disulphide bridge between A6Cys and A11Cys, and having insulin activity.

Without wishing to be bound by theory, it appears that, in the primary structure of the insulin molecule, a number of the amino acids in the B-chain are responsible for the polymerisation of insulin in clinical preparations. These include those in positions B12, B16 and B26. In particular, the replacement of Val by Thr in position B12 or Tyr by Ala in position B16 or B26 significantly reduces the tendency of the insulin analogues to polymerise even at high concentrations (see Example 9). This enhanced tendency to exist as a monomeric structure is not affected by deletion of either one or both of the terminal amino acids of the B-chain.

Description of the Invention

The Scheme, below, shows the construction of the expression plasmids pNHI-2/AOX1, pNHI-3/AOX1, pNHI-4/AOX1 and the engineering of recombinant cells YP99/NHI-2, YP99/NHI-3 and YP99/NHI-4. It sets out a representative procedure for the preparation of compounds of the invention, by analogy with the use of the human insulin target gene (HI) housed in the shuttle plasmid pHI/PGK. This shuttle vector is constructed from the plasmid pVT102-U (acquired from Canadian Research Institute) and subsequently multiplied by PCR (Maniatis *et al* (1989), Molecular Cloning A Laboratory Manual, 2nd ed. New York: Cold Spring Harbour Laboratory), to obtain multiple copies of human insulin target gene (HI) and flanking alpha mating factor leader (MFL) sequence. The target gene is then cloned into plasmid pPIC9 which is subsequently linearised with BglII prior to being employed to transform *P. pastoris* cell GS115 by the spheroplast method. Once plasmid pPIC9 containing the target gene is internalised, it integrates into the chromosomal DNA of the host cell [1]. Transformed cells bearing a high copy number of the HI gene are selected using the antibiotic G418 by the method described by Scover *et al* [2]. The presence of multiple copies of the HI are ascertained by the dot blotting method [3]. Cells bearing a high

copy number of the HI gene are utilised to generate the human insulin precursor by fermentation, and after purification converted to human insulin by tryptic transpeptidation.

In order to obtain recombinant forms of human insulin analogues according to this invention, target genes were produced. This was accomplished by the "gap double-stranded DNA" method described by Li Yiping *et al* [6] which permits site-directed mutations in the HI target gene. Primers specifically designed to give B12Thr, B16Ala and B26Ala were as follows;

For B12Thr (NHI-2): refer to Wang *et al, supra*

For B16Ala (NHI-3): 5' TGA GGC TTT GNN YTT GGT TTG CG 3' (SEQ ID No.1) in which N can be any nucleotide (G, A, T or C), and Y is C or G.

For B26Ala (NHI-4): 5' GAA AGA GGTT TTC NNY ACT CCT AGG GC 3' (SEQ ID No.2) in which N and Y are as defined above.

Novel human insulin analogues may be obtained by removing B30Thr and/or B1Phe, e.g. yielding a des-B1 and/or des-B30 analogue. Deletion may be achieved by known methodology. Rather than tryptic transpeptidation, to produce des-B30 human insulin, limited hydrolysis has been adopted, using trypsin in the preferred method, which further simplifies the process and increases the yield of insulin.

The methylotrophic yeast, *Pichia pastoris* is the preferred host for use in this invention for the preparation of insulin analogues because, as the Examples show, it has the advantages of high expression, simple processing, low production cost and high density culture. Furthermore it offers the advantages of a eukaryotic cell system; the correct folding and post-translational processing of secreted protein. These advantages greatly enhance the possibility of utilizing *P. pastoris* as the expression host in the scale-up of human insulin production. Its use in the expression of proteins of commercial importance has been documented elsewhere [3-5].

Human insulin analogues of the invention may be used in therapy. Their application and utility will be readily evident to those of ordinary skill in the art, e.g. in the treatment of diabetes mellitus.

Brief Description of the Drawings

Figure 1 shows the construction of pNHI-2/AOX1 plasmid of *Pichia pastoris*.

The following Examples illustrate the invention.

Example 1 Cloning of Mutated HI Gene

The plasmid pVT102-U from Canadian Biotechnology Research Institute was used to construct the plasmid pHI/PGK according to the standard method described

in Maniatis *et al* (1989). The construct pHI/PGK is a shuttle plasmid with phosphoglycerate kinase (PGK) promoter, followed by alpha mating factor leader sequence (MFL) to direct secretion of the product of the human insulin target gene (HI) flanked by a BamHI site at MFL 5' end and a HindIII site at HI 3' end. Using pHI/PGK as template, together with TCCGGATCCATGAGATTT (SEQ ID NO. 3) as the 5' primer and TGAATTCTTCTAGTTGCAGTAGTTT (SEQ ID NO. 4) as the 3' primer, DNA fragments containing MFL and HI with the BamHI site GGATCC at 5' end and the EcoRI site GAATTC at the 3' end were obtained by PCR. To obtain DNA fragments containing MFL and the target gene NHI-2 (B12Thr), NHI-3 (B16Ala) and NHI-4 (B26Ala) the HI target gene in pHI/PGK plasmid was first mutated by site-directed mutagenesis then replicated by PCR. By inserting these fragments behind the AOX1 promoter of the plasmid pPIC9 (Invitrogen), expression plasmids pNHI-2/AOX1, pNHI-3/AOX1 and pNHI-4/AOX1 were obtained (see the Scheme and the accompany drawing; the latter shows the first plasmid, and the others may be prepared by the same procedure). The primers used to obtain the mutated genes in this invention have SEQ ID NOS. 1, 2 and 3.

Example 2 Construction and Screening of Expression Cell

The expression plasmids were linearised by BglII and used to transform *P. pastoris* cell GS115 (Invitrogen) using the spheroplast method. The linearised plasmids, once internalized, integrate into the chromosomal DNA of the host cell [1]. The recombinant cells, designated YP99/NHI-2, YP99/NHI-3 and YP99/HNI-4 with high copy number of the target gene, were selected by antibiotic G418 [2] and identified by the dot blotting method [3].

Example 3 Preparation of Precursors of HI analogues

High density fermentation was carried out in a 15 litre fermenter [7]. The following salt solutions were used in the fermentation: BSM - H_3PO_4 26.7 ml/l, $\text{CaSO}_4 \cdot \text{H}_2\text{O}$ 0.93 g/l, K_2SO_4 18.2 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 14.9 g/l, KOH 4.13 g/l; PTM1 - $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 6 g/l, KI 0.08 g/l, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 3.0 g/l, $\text{NaMoO}_4 \cdot \text{H}_2\text{O}$ 0.2 g/l, H_3BO_3 0.02 g/l, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.5 g/l, ZnSO_4 20.0 g/l, H_2SO_4 5 ml/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 65.0 g/l.

Fermentation medium containing 6 L of salt solution BSM and 300 ml of glycerol is sterilised in the fermenter. Its pH is adjusted to 5.5 with 50% ammonium hydroxide. A 5 ml aliquot of salt solution PTM1 containing 1 mg of biotin is added per 1 litre of culture medium. The expression cell is inoculated to 50 ml YPG and grown in a shake flask at 30°C for 24 hr. The broth is added to 600 ml of YPG, shaken in 35 flasks for 24 hr, added to the culture medium and fermented for 24 hr to deplete

glycerol. Methanol solution containing PTM1 (5 ml/l) and biotin (1 mg/l) is added to induce the expression. The inductive fermentation is continued for 84 hr by feeding the above methanol solution. During the fermentation, the pH is maintained at 5.5 by adding 50% ammonium hydroxide. The expression level is measured by radioimmunoassay, SDS-polyacrylamide gel electrophoresis [8] and HPLC.

Example 4 Separation and Purification of the Precursors

The fermentation broth is centrifuged to remove the cell bodies. The supernatant is applied to a C8 column and purified by HPLC. After a single step of purification, a product can be obtained that is homogeneous in native polyacrylamide gel electrophoresis.

Example 5 Transpeptidation of the Precursors

Purified precursors of HI analogues from Example 4 are dissolved in DMSO/1,4-butanediol/H₂O (15:70:15, v/v) to a concentration of 30 mg/ml. Thr(Bu^t)-OBu^t is added in excess, and the pH is adjusted to 6.5 by ammonium hydroxide. TPCK-trypsin is added (substrate:enzyme = 5:1) and the reaction mixture is incubated at 25°C for 6 hr. The reaction is stopped by acidification. The product is precipitated using acetone, and purified by HPLC using C8 column.

Example 6 Preparation of des-B30 analogues

Purified precursors of HI analogues are dissolved in pH 8, 0.1M ammonium bicarbonate to a concentration of 10 mg/ml. TPCK-trypsin is added (substrate:enzyme = 200:1) and the reaction mixture is incubated at 25°C overnight. The product is analysed by native polyacrylamide gel electrophoresis.

Example 7 Preparation of des-B1 analogues

HI analogues are reacted with phenylisocyanate in a molar ratio of 1:2, prior to treatment with trifluoroacetic acid as described by Bradenburg & Hoppe-Seyler, Physiol. Chem. 350:471. The products of this reaction are separated and analysed by electrophoresis and found to be almost exclusively des-B1 forms of insulin analogues.

Example 8 Preparation of des-B1, des-B30 analogues

Prepared by processing precursors of HI analogues as described in Example 6 followed sequentially by that described in Example 7.

Example 9 Determination of structural forms

The structural form of the recombinant human insulin analogues prior to deletion of the one or both terminal amino acids of the B-chain is determined electrophoretically. A preparation of each analogue is passed through Superdex G-75 column (HR 10/30). HI and [B28Lys, B29Pro] insulin (Lispro) are used as negative

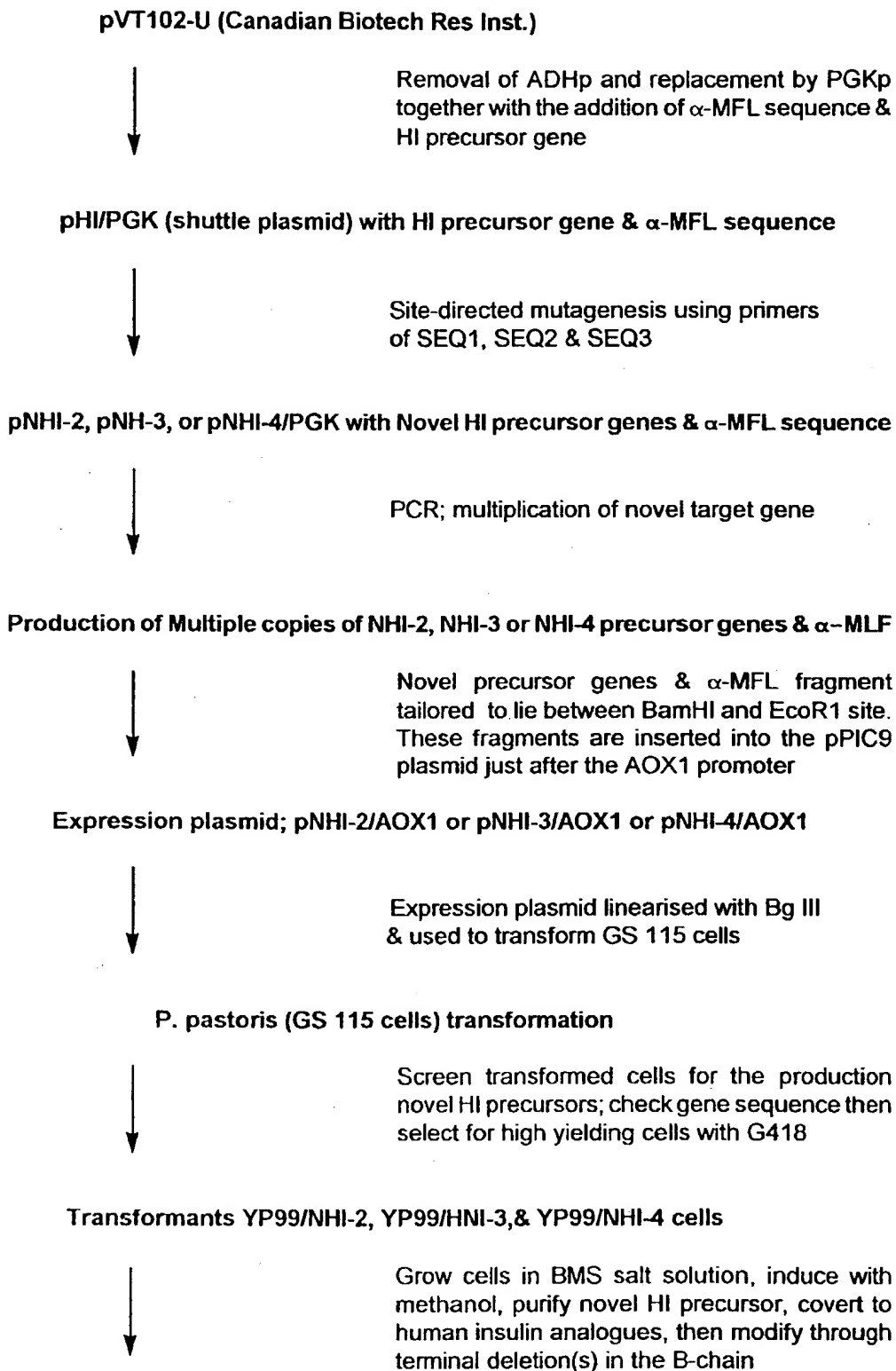
and positive controls respectively. Phosphate buffered saline pH 7.4 is used as an elution buffer and the flow rate fixed at 0.4 ml/min. The concentration of the sample preparation is 1.2 mg/ml. The retention times and the peak profiles of human insulin analogues are shown in the following Table.

Sample	Retention Time, min	Peak profile
HI	36.4	Unsymmetrical
[B28Lys, B29Pro]HI	39.4	Symmetrical
[B12Thr]HI	39.4	Symmetrical
[B16Ala]HI	38.3	Symmetrical
[B26Ala]HI	38.9	Symmetrical

These results demonstrate that HI analogues B12Thr, B16Ala and B26Ala are all monomeric in form. They have a similar retention time and peak profile as the known positive control [B28Lys, B29Pro] human insulin.

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Scheme

CLAIMS

1. An insulin analogue wherein the 12th, 16th or 26th amino acid of the B chain of human insulin (Val, Tyr or Tyr, respectively) is substituted by another amino acid, such that the analogue is monomeric, and which also comprises a deletion at B1(Phe)
5 and/or B30 (Thr).
2. An insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Val, Tyr or Tyr, respectively) is substituted by another amino acid, such that the analogue is monomeric, for therapeutic use.
3. An insulin analogue according to claim 1, wherein the 12th amino acid is
10 substituted by Thr (B12Thr).
4. An insulin analogue according to claim 1 or claim 2, wherein the 16th amino acid is substituted by Ala (B16Ala).
5. An insulin analogue according to claim 1 or claim 2, wherein the 26th amino acid is substituted by Ala (B26Ala).
- 15 6. An insulin analogue according to claim 3, which comprises a deletion at B1 (des-B1, B12Thr).
7. An insulin analogue according to claim 3, which comprises a deletion at B30 (des-B30, B12Thr).
8. An insulin analogue according to claim 3, which comprises deletions at B1
20 and B30 (des-B1, des-B30, B12Thr).
9. An insulin analogue according to claim 4, which comprises a deletion at B1 (des-B1, B16Ala).
10. An insulin analogue according to claim 4, which comprises a deletion at B30 (des-B30, B16Ala).
- 25 11. An insulin analogue according to claim 4, which comprises deletions at B1 and B30 (des-B1, des-B30, B16Ala).
12. An insulin analogue according to claim 4, which comprises a deletion at B1 (des-B1, B26Ala).
13. An insulin analogue according to claim 5, which comprises a deletion at B30
30 (des-B30, B16Ala).
14. An insulin analogue according to claim 5, which comprises deletions at B1 and B30 (des-B1, des-B30, B26Ala).

1/1

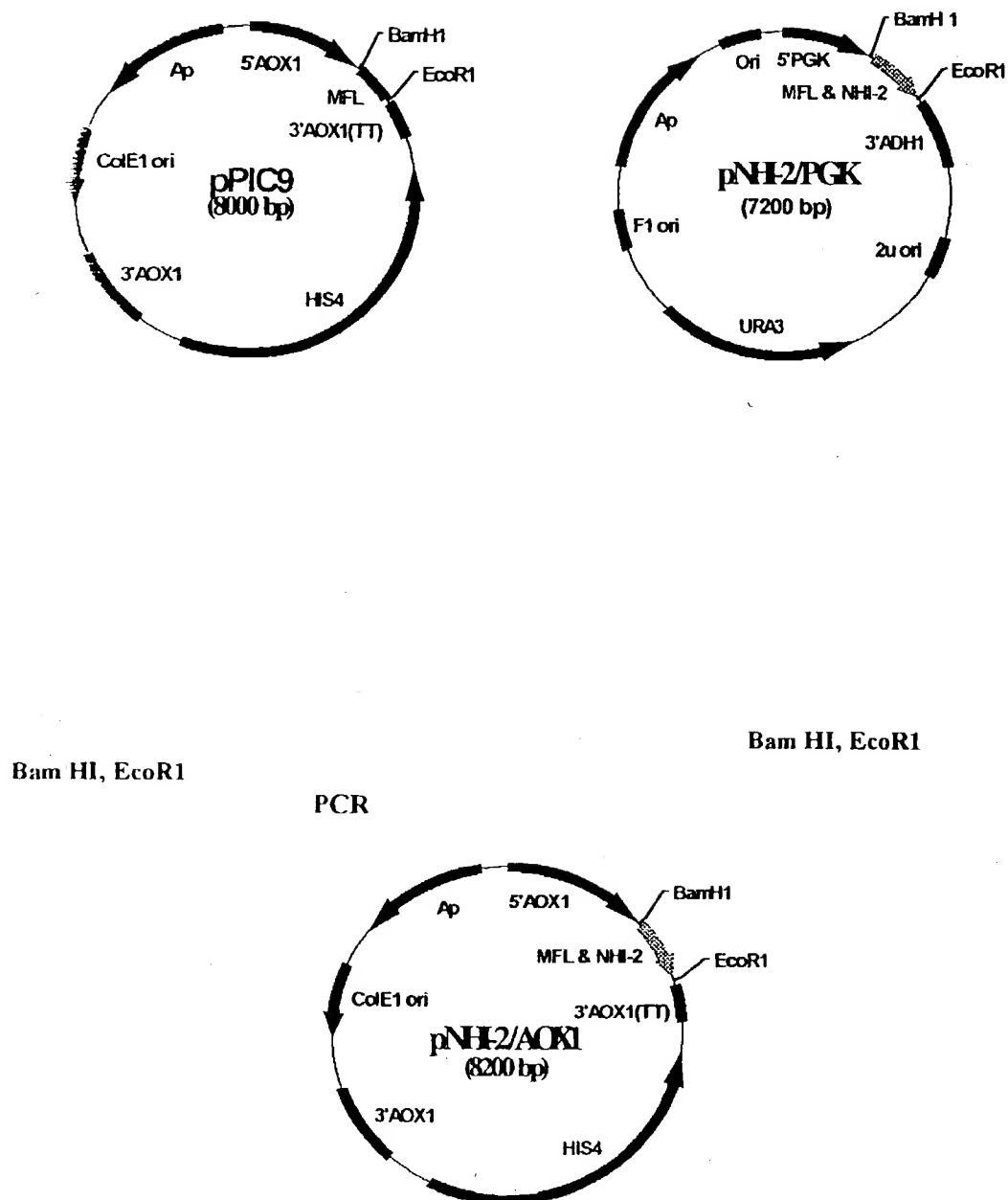


Figure 1

SEQUENCE LISTING

<110> Shanghai Institute of Biotechnology, et al

<120> MONOMERIC ANALOGUES OF HUMAN INSULIN

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<141> 2000-09-08

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/62 A61P3/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KRISTENSEN, CLAUS ET AL: "Alanine scanning mutagenesis of insulin" J. BIOL. CHEM. (1997), 272(20), QQ12978-12983, 16 May 1997 (1997-05-16), XP002141113 page 12981, left-hand column, paragraph 2 -page 12983, left-hand column, paragraph 1; table I -/-	2,4,5



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

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INTERNATIONAL SEARCH REPORT

Intern: .al Application No

PCT/GB 00/03460

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 125, no. 21, 18 November 1996 (1996-11-18) Columbus, Ohio, US; abstract no. 266145, WANG, QIONG-QING ET AL: "Studies on receptor binding site of insulin: the hydrophobic B12Val can be substituted by hydrophilic Thr" XP002141114 cited in the application abstract & BIOCHEM. MOL. BIOL. INT. (1996), 39(6), 1245-1254, 1996,	1,3
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